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Applicant(s):
Demandeur(s):
INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE (INSERM)
75654 Paris Cédex 13
FRANCE
Bayer Pharma
92807 Puteaux Cedex
FRANCE
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EUROPEAN PATENT APPLICATION

entitled : CATALYTIC ANTI-FACTOR VIII ALLO-ANTIBODIES.

Applicants : INSERM
BAYER PHARMA

CATALYTIC ANTI-FACTOR VIII ALLO-ANTIBODIES.

FIELD OF THE INVENTION

- 5 The present invention relates to a method of determining the presence of catalytic anti-Factor VIII allo-antibodies capable of degrading Factor VIII in a mammal, and of characterising the cleavage sites in said Factor VIII molecule by said catalytic anti-Factor VIII allo-antibodies.
- 10 The present invention also relates to an anti-Factor VIII allo-antibody-catalysed Factor VIII degradation inhibitor.

15 The present invention further relates to a pharmaceutical composition comprising said catalytic anti-Factor VIII allo-antibodies which are capable of degrading Factor VIII and which originate from said method of determination, and to a pharmaceutical composition comprising said anti-Factor VIII allo-antibody-catalysed Factor VIII degradation inhibitor.

20 Finally, the present invention relates to the application in therapeutics of said anti-Factor VIII allo-antibody-catalysed Factor VIII degradation inhibitor, of a pharmaceutical composition comprising said catalytic anti-Factor VIII allo-antibodies which are capable of degrading Factor VIII and which originate from said method of determination, and of a pharmaceutical composition comprising said anti-Factor VIII allo-antibody-catalysed Factor VIII
25 degradation inhibitor.

BACKGROUND TO THE INVENTION

30 Haemophilia A is an X chromosome-linked recessive disorder resulting in defective or deficient Factor VIII molecules, which, in its severe form, is a life-threatening and crippling haemorrhagic disease.

Infusion of homologous Factor VIII to patients with severe haemophilia A results, in 25% of the cases, in the emergence of anti-Factor VIII allo-antibodies (Ehrenforth, S., Kreuz, W., Scharrer, I., Linde, R., Funk, M.,
5 GÜNGÖR, T., Krackhardt, B. and Kornhuber, B., « *Incidence of development of factor VIII and factor IX inhibitors in haemophiliacs* », Lancet, 1992, 339: 594-598), that inhibit Factor VIII procoagulant activity by steric hindrance of the interaction of Factor VIII either with stabilising molecules (Saenko, E. L., Shima, M., Rajalakshmi, K. J. and Scandella, D., « *A role for the C2 domain*
10 *of factor VIII in binding to von Willebrand factor* », J. Biol. Chem., 1994, 269: 11601-11605 ; and Saenko, E. L., Shima, M., Gilbert, G. E., and Scandella, D., « *Slowed release of thrombin-cleaved factor VIII from von Willebrand factor by a monoclonal and a human antibody is a novel mechanism for factor VIII inhibition* », J. Biol. Chem., 1996, 271: 27424-
15 27431), with molecules essential for its activity (Arai, M., Scandella, D., and Hoyer, L. W., « *Molecular basis of factor VIII inhibition by human antibodies : Antibodies that bind to the factor VIII light chain prevent the interaction of factor VIII with phospholipid* », J. Clin. Invest., 1989, 83: 1978-1984 ; and Zhong, D., Saenko, E. L., Shima, M., Felch, M. and Scandella,
20 D., « *Some human inhibitor antibodies interfere with factor VIII binding to Factor IX* », Blood, 1998, 92: 136-142), or with activating molecules (Lubahn, B. C., Ware, J., Stafford, D. W., and Reiser, H. M., « *Identification of a FVIII epitope recognized by a human hemophilic inhibitor* », Blood, 1989, 73: 497-499 ; and Neuenschwander, P. F., and Jesty, J., « *Thrombin-activated and*
25 *factor Xa-activated human factor VIII : differences in cofactor activity and decay rate* », Arc. Biochem. Biophys., 1992, 296: 426-434).

SUMMARY OF THE INVENTION

30 In an entirely surprising way, a discovery has been made by the Applicants of a degradation of Factor VIII by allo-antibodies of two high responder patients with severe haemophilia A, demonstrating a heretofore unknown mechanism

by which Factor VIII inhibitors may prevent the pro-coagulant function of Factor VIII.

5 The Applicant's discovery of catalytic anti-Factor VIII allo-antibodies is to the best of his knowledge the first report on the emergence of catalytic antibodies that are INDUCED upon treatment of patients with Factor VIII. It was heretofore considered very surprising, even absurd or unbelievable, that antibodies are formed, in the presence of Factor VIII, which would actually render the Factor VIII molecule inactive through catalytic hydrolysis
10 (« proteolysis »). However, the catalytic antibodies reported so far, are all auto-antibodies found in the course of a disease process or in physiological conditions. Thus, induced antibodies are called ALLO-antibodies, the origin of which is clearly different from the origin of AUTO-antibodies in any auto-immune disease.

15 The calculated average K_m and apparent V_{max} for the reaction of anti-Factor VIII antibodies of one of the patients were $9.46 \pm 5.62 \mu M$ and $85 \pm 60 \text{ fmol.min}^{-1}$, respectively. The kinetic parameters of Factor VIII hydrolysis suggest a functional role for the catalytic immune response in the inactivation
20 of Factor VIII *in vivo*.

The characterisation of anti-Factor VIII allo-antibodies as site-specific proteases hence provide new approaches to the treatment of diseases of a patient who possess anti-Factor VIII allo-antibodies.

25 Thus, according to a first aspect, the present invention provides a method of determining the presence of catalytic anti-Factor VIII allo-antibodies capable of degrading Factor VIII in a mammal, characterised in that it comprises :

30 i) isolating the plasma from a sample of blood taken from said mammal,

ii) isolating anti-Factor VIII allo-antibodies from said plasma ;

5 iii) placing said anti-Factor VIII allo-antibodies in contact with Factor VIII for a period of time sufficient to permit any degradation of said Factor VIII by said anti-Factor VIII allo-antibodies ; and

10 iv) determining, after said period of time, whether said Factor VIII has effectively been degraded by said anti-Factor VIII allo-antibodies.

According to an embodiment of step ii) of the method of the present invention, said anti-Factor VIII allo-antibodies are isolated from said plasma by combining them with said Factor VIII, said Factor VIII being preferably coupled to a matrix. Advantageously, in step ii), said anti-Factor VIII allo-
15 antibodies are isolated by affinity chromatography. Preferably, in step ii), said affinity chromatography comprises the use of a Sepharose matrix, preferably activated with cyanogen bromide.

According to an embodiment of step iii) of the method of the present invention, said Factor VIII is labelled with a labelling agent, preferably a
20 radio-labelling agent, such as ^{125}I in particular. Advantageously, in step iii), said Factor VIII is placed in contact with the anti-Factor VIII allo-antibodies for a period of time of between about 0.5 and about 30 hours, preferably about 10 hours, at a temperature of about 15 to about 40°C, preferably 38°C.

25 According to an embodiment of step iv) of the method of the present invention, the determination of whether said Factor VIII has effectively been degraded by said anti-Factor VIII allo-antibodies is carried out by a determination comprising a separation technique, such as gel electrophoresis,
30 such as SDS PAGE in particular, or gel filtration, such as fast protein liquid chromatography gel filtration in particular, and a visualisation technique, such as autoradiography in particular.

In accordance with a further embodiment of the method of the present invention, said method is characterised in that it further comprises :

- 5 v) characterising the site(s) in said Factor VIII molecule cleaved by said anti-Factor VIII allo-antibodies.

According to an embodiment of step v) of the method of the present invention, said characterisation is carried out by placing said Factor VIII in contact with
10 said anti-Factor VIII allo-antibodies capable of degrading Factor VIII, separating and then sequencing the fragments of Factor VIII resulting therefrom. Advantageously, said separation is carried out using a technique such as gel electrophoresis, such as SDS PAGE in particular, or gel filtration. Said sequencing is advantageously carried out using a technique such as N-
15 terminal sequencing, such as by using an automatic protein microsequencer in particular. By using the said sequencing, the following scissile bonds are located : Arg³⁷²-Ser³⁷³, located between the A1 and A2 domains, Tyr¹⁶⁸⁰-Asp¹⁶⁸¹, located on the N-terminus of the A3 domain, and Glu¹⁷⁹⁴-Asp¹⁷⁹⁵ located within the A3 domain of the Factor VIII molecule.

20 According to a second aspect, therefore, the present invention provides an amino acid sequence :

Ser Val Ala Lys Lys His Pro ;

25 an amino acid sequence :

Asp Glu Asp Glu Asn Gln Ser ; and

30 an amino acid sequence :

Asp Gln Arg Gln Gly Ala Glu .

The present invention also extends to variants or analogues of this or any other sequence of Factor VIII which are capable of inhibiting any site in the Factor VIII molecule which is susceptible to being lysed by an anti-Factor VIII
5 allo-antibody. Within the context of the present invention, such a variant can be, for example, a peptide or non-peptide analogue of an amino acid sequence described *supra* which inhibits any site in the Factor VIII molecule which is susceptible to being lysed by an anti-Factor VIII allo-antibody. Such a variant
10 can be, for example, a variant of the sequence which is either shorter by a few amino acids, at the N-terminal, the C-terminal, or both termini, for example, or longer by a few amino acids (it being possible to obtain such variants by chemical synthesis or by enzymatic digestion of the naturally occurring molecule), so long as the variant inhibits any site in the Factor VIII molecule
15 which is susceptible to being lysed by an anti-Factor VIII allo-antibody.

Hence, according to a third aspect, the present invention provides an anti-Factor VIII allo-antibody-catalysed Factor VIII degradation inhibitor. Advantageously, this inhibitor is characterised in that it comprises a protease
20 inhibitor. Examples of protease inhibitors that can be used as anti-Factor VIII allo-antibody-catalysed Factor VIII degradation inhibitors within the context of the present invention, without being limited thereto, are fluorophosphate-type inhibitors, such as DFP for example, or sulphonyl fluoride-type inhibitors, such as PMSF or AEBSF (4-(2-aminoethyl)benzenesulphonyl
25 fluoride hydrochloride (notably marketed by Roche Diagnostics GmbH, Mannheim, Germany, under the trademark Pefabloc[®])), for example. More particularly, this inhibitor is characterised in that said inhibitor inhibits cleavage of the scissile bonds : Arg³⁷²-Ser³⁷³, located between the A1 domains, Tyr¹⁶⁸⁰-Asp¹⁶⁸¹, located on the N-terminus of the A3 domain, and Glu¹⁷⁹⁴-
30 Asp¹⁷⁹⁵ located within the A3 domain of the Factor VIII molecule. More

preferably still, this inhibitor is characterised in that it comprises a peptide or non-peptide analogue of the amino acid sequence :

Ser Val Ala Lys Lys His Pro ;

5

a peptide or non-peptide analogue of the amino acid sequence :

Asp Glu Asp Glu Asn Gln Ser ; or

10 a peptide or non-peptide analogue of the amino acid sequence :

Asp Gln Arg Gln Gly Ala Glu .

15 The Factor VIII degradation inhibitors as defined *supra*, as well as their addition salts, in particular their pharmaceutically acceptable addition salts, have a very valuable pharmacological profile in that they possess neutralising activity towards anti-Factor VIII allo-antibodies.

20 These properties justify their application in therapeutics and the invention further relates, by way of drugs, to the Factor VIII degradation inhibitors above, as well as their addition salts, in particular their pharmaceutically acceptable addition salts.

25 They will therefore be particularly indicated in the treatment of diseases of, *inter alia*, haemophilic nature, more particularly diseases involving coagulation defects due to Factor VIII insufficiency.

30 An example of their use which may be mentioned is the treatment of high responder patients with diseases such as mild or severe haemophilia A, for example (in the case in which catalytic antibodies are found in these patients), on the one hand, and/or, on the other hand, patients suffering from auto-

immune diseases for example (in the case in which catalytic antibodies are found in these patients).

Thus, according to a fourth principal aspect, the present invention provides a
5 solution to a long-felt need through a pharmaceutical composition characterised in that it comprises a pharmaceutically effective amount of at least one anti-Factor VIII allo-antibody capable of degrading Factor VIII, as defined *supra*, notably as obtainable from the method described *supra*, or one of its pharmaceutically acceptable addition salts incorporated in a
10 pharmaceutically acceptable excipient, vehicle or carrier.

Further, according to a fifth principal aspect, the present invention provides a pharmaceutical composition characterised in that it comprises a pharmaceutically effective amount of at least one Factor VIII degradation
15 inhibitor, as defined *supra*, or one of its pharmaceutically acceptable addition salts incorporated in a pharmaceutically acceptable excipient, vehicle or carrier.

These compositions can be administered by the buccal, rectal, parenteral,
20 transdermal, ocular, nasal or auricular route, for example.

These compositions can be solid or liquid and can be presented in the pharmaceutical forms commonly used in human medicine, such as, for example, simple or coated tablets, gelatine capsules, granules, suppositories,
25 injectable preparations, transdermal systems, eye drops, aerosols and sprays, and ear drops. They are prepared by the customary methods. The active principle, which consists of a pharmaceutically effective amount of at least one Factor VIII degradation inhibitor as defined *supra*, or one of its pharmaceutically acceptable addition salts can be incorporated therein
30 together with excipients normally employed in pharmaceutical compositions, such as talc, gum Arabic, lactose, starch, magnesium stearate, polyvidone, cellulose derivatives, cocoa butter, semi-synthetic glycerides, aqueous or non-

aqueous vehicles, fats of animal or vegetable origin, glycols, various wetting agents, dispersants or emulsifiers, silicone gels, certain polymers or copolymers, preservatives, flavourings and colours. The preferred pharmaceutical form is an injectable form.

5

The invention also covers a pharmaceutical composition with neutralising activity which can be used especially as a favourable treatment of diseases such as haemophilia A with production of anti-Factor VIII allo-antibodies ; autoimmune diseases with anti-Factor VIII allo-antibodies (in case catalytic antibodies are found in these patients) in particular, said composition being characterised in that it comprises a pharmaceutically effective amount of at least one Factor VIII degradation inhibitor above, or one of its pharmaceutically acceptable addition salts incorporated in a pharmaceutically acceptable excipient, vehicle or carrier.

15

The invention also covers a method of therapeutic treatment of a mammal suffering from a pathology resulting from the level of Factor VIII in the blood thereof, characterised in that a therapeutically effective amount of at least one Factor VIII degradation inhibitor as defined *supra* or one of its pharmaceutically acceptable addition salts is administered to the said mammal.

20

This method affords especially a favourable treatment of diseases of haemophilic nature, in particular a pathology resulting from a lack of Factor VIII in the blood thereof.

25

The invention also covers a pharmaceutical composition with anti-thrombotic activity which can be used especially as a favourable treatment of diseases such as thrombosis in particular, said composition being characterised in that it comprises a pharmaceutically effective amount of at least one anti-Factor VIII allo-antibody capable of degrading Factor VIII, notably as obtainable from the method described above, or one of its pharmaceutically acceptable

30

addition salts incorporated in a pharmaceutically acceptable excipient, vehicle or carrier.

5 The invention also covers a method of therapeutic treatment of mammals, characterised in that a therapeutically effective amount of at least one anti-Factor VIII allo-antibody as defined *supra* or one of its pharmaceutically acceptable addition salts is administered to the said mammal.

10 This method affords especially a favourable treatment of diseases of thrombotic nature, in particular said pathology resulting from the presence of an excess of Factor VIII in the blood thereof.

15 In human and animal therapeutics, the anti-Factor VIII allo-antibodies or the Factor VIII degradation inhibitors as defined *supra* can be administered by themselves or in association with a physiologically acceptable excipient, in any form, in particular orally in the form of gelatine capsules or tablets, or parenterally in the form of injectable solutions. It is possible to envisage other forms of administration such as suppositories, ointments, creams, gels or aerosol preparations.

20

Within the context of the present invention, the following terms are used :

25 « catalytic anti-Factor VIII allo-antibodies », which is understood as meaning antibodies directed to Factor VIII endowed with a catalytic activity induced in haemophilia A patients upon transfusion with therapeutic preparations of Factor VIII ;

« Factor VIII », which is understood as meaning a co-enzyme of Factor IX in the enzymatic cleavage of Factor X during the blood coagulation process ;

30

« degradation of Factor VIII », which is understood as meaning the generation of fragments from Factor VIII that do not appear due to a spontaneous

hydrolysis, or due to hydrolysis by physiologically cleaving enzymes, *i.e.* thrombin, activated Factor IX, activated Factor X, and activated protein C ;

- 5 « anti-Factor VIII allo-antibody-catalysed Factor VIII degradation inhibitor », which is understood as meaning any peptide, belonging or not to the Factor VIII sequence, or protease inhibitor that are capable of specifically neutralising the hydrolysing activity of anti-Factor VIII catalytic antibodies ;

DESCRIPTON OF A PREFERRED EMBODIMENT OF THE PRESENT INVENTION

10

- Human recombinant Factor VIII was radio-labelled with ^{125}I . Anti-Factor VIII allo-antibodies were affinity-purified from the plasma of three haemophilia patients with inhibitor on a Sepharose matrix to which immunopurified human Factor VIII had been coupled. Affinity-purified anti-Factor VIII antibodies of patients Bor, Che and Wal inhibited Factor VIII pro-coagulant activity up to 57.0, 64.0 and 43.0 BU/mg of IgG, respectively.

- Co-incubation of labelled Factor VIII with the anti-Factor VIII allo-antibodies resulted, in the case of two patients out of three, in the proteolysis of the molecule. The specificity of the hydrolysis on the antibody combining sites of anti-Factor VIII allo-antibodies of the IgG isotype was demonstrated. Co-incubation of [^{125}I]-Factor VIII with affinity-purified anti-Factor VIII IgG of patients Bor and Wal in the presence of the protease inhibitors aprotinin (0.15 μM), E-64 (28 μM), EDTA (1.3 μM), leupeptin (10 μM), and pepstatin (10 μM) did not result in inhibition of proteolytic activity.

- The Applicants have characterised the major cleavage sites for catalytic IgG in the Factor VIII molecule, to be as follows: Arg³⁷² - Ser³⁷³, located between the A1 and A2 domains of Factor VIII ; Tyr¹⁶⁸⁰ - Asp¹⁶⁸¹, located on the N-terminus of the A3 domain ; and Glu¹⁷⁹⁴ - Asp¹⁷⁹⁵ located within the A3 domain.

The time and dose-dependency of the hydrolysis of Factor VIII by anti-Factor VIII allo-antibodies has been demonstrated. In particular, hydrolysis was observed under conditions where anti-Factor VIII IgG and Factor VIII were co-incubated at molar ratios that were 80- to 9500-fold lower than those expected to be present in patients' plasma, suggesting that hydrolysis is a mechanism of Factor VIII inactivation by the patients' allo-antibodies *in vivo*.

The Applicants have further investigated the kinetics of antibody-mediated hydrolysis of Factor VIII by incubating anti-Factor VIII IgG of patient Wal with increasing concentrations of unlabeled Factor VIII in the presence of a fixed concentration of [¹²⁵I]-Factor VIII. The curves of the reciprocal of the velocity plotted as a function of the reciprocal of the substrate concentration were linear ($r=0.99$), suggesting that the reaction conformed to simple Michaelis-Menten kinetics, as already observed for polyclonal catalytic auto-antibodies. The apparent catalytic efficiency, V_{max} and rate of hydrolysis of anti-Factor VIII allo-antibodies were calculated in the case of patient Wal. The kinetic parameters of hydrolysis calculated *in vitro*, suggest that proteolysis may be a mechanism of Factor VIII inactivation by patients' allo-antibodies *in vivo*.

The association of Factor VIII with von Willebrand Factor (vWF) increases the catalytic rate of thrombin for Factor VIII, whereas it protects Factor VIII from hydrolysis by activated protein C (APC). The addition of vWF to Factor VIII resulted in partial inhibition of hydrolysis of Factor VIII by anti-Factor VIII IgG, *i.e.* 36.9%, when purified vWF and Factor VIII were mixed using a wt/wt ratio similar to that present in normal plasma, *i.e.* 30 µg/ml of vWF versus 300 ng/ml of Factor VIII.

The identification of anti-Factor VIII allo-antibodies as catalytic antibodies extends the spectrum of catalytic immune responses, in addition to previous reports of hydrolysing antibodies against vasoactive intestinal peptide (VIP) in

asthma patients, DNA-hydrolysing antibodies in patients with SLE and thyroglobulin-specific catalytic antibodies in patients with autoimmune thyroiditis. This is also the first report to the knowledge of the Applicants of the induction of a catalytic antibody in the human, in response to exogenous administration of a protein antigen. The kinetic parameters of Factor VIII hydrolysis by anti-Factor VIII IgG exhibiting catalytic properties and the estimated amounts of these antibodies in plasma, suggest a functional role for the catalytic immune response in inactivating Factor VIII *in vivo*. Within a polyclonal mixture of anti-Factor VIII allo-antibodies which differ in their functional properties, catalytic antibodies may inhibit Factor VIII pro-coagulant activity at faster rates than non-catalysing anti-Factor VIII antibodies. Identification of peptide epitopes that are the targets for proteolytic anti-Factor VIII antibodies may thus be critical for our understanding of the pathophysiology of the Factor VIII inhibitor response. Furthermore, the characterisation of Factor VIII inhibitors as site-specific proteases will provide new approaches to the treatment of patients possessing anti-Factor VIII allo-antibodies.

BRIEF DESCRIPTION OF THE FIGURES

The invention will be better understood and other objects, characteristics and advantages thereof will become more clearly apparent from the following explanatory description referring to the attached Figures, which are given solely by way of non-limiting Examples illustrating the specificity of the cleavage of Factor VIII by anti-Factor VIII allo-antibodies.

Figure 1: Hydrolysis of [¹²⁵I]-Factor VIII by affinity-purified anti-Factor VIII IgG antibodies of haemophilia A patients with inhibitor

Figure 1(A):

[¹²⁵I]-labelled Factor VIII was incubated with affinity-purified anti-Factor VIII IgG of patients Bor (lane Bor), Che (lane Che) and Wal (lane Wal), or

with buffer alone (lane 1) for 10 h at 38°C prior to SDS-PAGE and autoradiography. In two of the three patients (Bor and Wal), incubation of Factor VIII with affinity-purified anti-Factor VIII IgG resulted in hydrolysis of the Factor VIII molecule. In contrast, the migration profile of Factor VIII was unchanged when [¹²⁵I]-labelled Factor VIII was incubated with anti-Factor VIII IgG purified from the plasma of patient Che (lane Che). The migration profile of Factor VIII was also unchanged upon incubation with human monoclonal M061 anti-digoxin IgG (mAb) or with normal unfractionated polyclonal human IgG (Sandoglobulin® , IVIg) that exhibit no inhibitory activity to Factor VIII.

Figure 1(B):

Flow-throughs of the affinity columns were devoid of anti-Factor VIII antibodies as determined by ELISA, and did not hydrolyse [¹²⁵I]-labelled Factor VIII.

Figure 1(C):

Removal of IgG from the acid eluates containing affinity-purified anti-Factor VIII antibodies of patients Wal and Bor by chromatography on protein G, resulted in the loss of their hydrolytic activity to Factor VIII.

Figure 2: Size exclusion chromatography of the catalytic activity of anti-Factor VIII antibodies

Figure 2(A):

To further exclude the possibility that the proteolytic activity of the antibodies was due to contaminating proteases, affinity-purified anti-Factor VIII antibodies of patient Wal were treated with 8 M urea and subjected to size

exclusion chromatography. A major peak was isolated in fraction 25 that corresponded to IgG as indicated by ELISA. The hydrolysing activity co-eluted with the IgG fraction and that the activity was not detected in fractions in which IgG was not present (*e.g.*, fraction 35).

5

Figure 2(B):

The major peak that was isolated in fraction 25 corresponded to IgG as indicated by SDS-PAGE of the radio-labelled content of the fraction.

10

Figure 3: Dose- and time-dependency of proteolysis of [¹²⁵I]-Factor VIII by affinity-purified anti-Factor VIII antibodies of haemophilia A patients with inhibitor.

15 The kinetics of the hydrolysis of Factor VIII by anti-Factor VIII allo-antibodies of patients Bor and Wal. The rate of hydrolysis of [¹²⁵I]-labelled Factor VIII by anti-Factor VIII IgG of patient Wal was faster than that exhibited by anti-Factor VIII IgG of patient Bor, suggesting either that catalytic antibodies of the patients exhibit different kinetic properties, or,
20 alternatively, that the proportion of catalytic antibodies among the anti-Factor VIII antibodies differ between the patients.

Figure 4: Hydrolysis of [¹²⁵I]-Factor VIII by anti-Factor VIII IgG antibodies in the presence of increasing amounts of cold Factor VIII

25

Kinetics of antibody-mediated hydrolysis of Factor VIII by incubating anti-Factor VIII IgG of patient Wal with increasing concentrations of unlabelled Factor VIII in the presence of a fixed concentration of [¹²⁵I]-Factor VIII. The addition of increasing amounts of unlabelled Factor VIII resulted in
30 dose-dependent inhibition of hydrolysis of [¹²⁵I]-Factor VIII by anti-Factor VIII IgG. Saturation of Factor VIII hydrolysis was not attained with the maximum concentration of Factor VIII that was used (*i.e.* 1.7 μ M). The

curves of the reciprocal of the velocity plotted as a function of the reciprocal of the substrate concentration were linear ($r=0.99$), suggesting that the reaction conformed to simple Michaelis-Menten kinetics, as already observed for polyclonal catalytic auto-antibodies.

5

Figure 5: Inhibition of catalytic activity of anti-Factor VIII IgG of patient Wal

The proteolysis of radio-labelled Factor VIII by the anti-Factor VIII allo-antibodies of patient Wal was inhibited to about 62% when the antibodies and Factor VIII were co-incubated in the presence of Pefabloc® (marketed by Roche Diagnostics GmbH, Mannheim, Germany), indicating the potency of certain serine protease inhibitor to neutralise the catalytic activity of some of the catalytic antibodies.

15

EXAMPLES

Example I: Affinity-purification of anti-Factor VIII antibodies

Antibodies were isolated from plasma by ammonium sulphate precipitation. Antibodies reactive with Factor VIII were then affinity-purified on a CNBr-activated Sepharose 4B matrix to which immuno-purified commercial human plasma-derived Factor VIII had been coupled (25000 U/3 g of gel). The flow-throughs of the columns were collected. After extensive washing with PBS pH 7.4, anti-Factor VIII antibodies were eluted using 0.2 M glycine pH 2.8, dialysed against PBS and concentrated with Centriprep. Flow-throughs and eluates were aliquoted and stored at -20°C until use. F(ab')₂ fragments of anti-Factor VIII antibodies were prepared as previously described.

The concentration of anti-Factor VIII IgG was 130, 20 and 280 µg per 10 mg of IgG applied to the column in the case of patients Bor, Che and Wal,

respectively, (*i.e.*, 143 ± 130 $\mu\text{g}/\text{mL}$ of unfractionated plasma), which is in agreement with previous observations.

Example II: Factor VIII-neutralising activity

5

The Factor VIII-neutralising activity of anti-Factor VIII antibodies was determined by the method of Kasper *et al.* and expressed as Bethesda units (BU) (ref). BU were defined as the inverse of the concentration of IgG which causes 50% inhibition of Factor VIII procoagulant activity. Residual Factor
10 VIII activity was measured in a one-stage assay by determination of the activated partial thromboplastin time using human plasma depleted of Factor VIII (Behring) as substrate and human placental pathromtin® (Behring) as activators. Heated plasma or immunopurified anti-Factor VIII IgG to be tested, were incubated with pooled citrated human plasma for 2 h at 37°C.
15 The clotting time of four serial dilutions of a reference plasma pool (Immuno AG, Wien) was compared with the clotting time of three dilutions of each sample to be tested. Dilutions were carried out in Owren-Koller buffer (Diagnostica Stago). Inter-assay variation ranged between 1 and 2.5%.

20 Affinity-purified anti-Factor VIII antibodies of patients Bor, Che and Wal inhibited Factor VIII pro-coagulant activity up to 57.0, 64.0 and 43.0 BU/mg of IgG, respectively.

Example III: Assay for hydrolysis of Factor VIII

25

Commercial human recombinant Factor VIII was labelled with ^{125}I to a specific activity of 11.6 nCi/ μg , by using the iodogen method. [^{125}I]-Factor VIII (1.5 to 150 ng) was incubated in 50 μl of 50 mM tris-HCl pH 7.7, 100 mM glycine, 0.025% Tween-20 and 0.02% NaN_3 alone or with 17 to 1667 nM
30 of immuno-purified anti-Factor VIII IgG for 5 min to 10 hours at 38°C. Human monoclonal anti-digoxin IgG M061 (mAb) and normal

unfractionated human polyclonal IgG (IVIg, Sandoglobulin®), were used as negative controls. Samples were mixed 1:1 with Laemmli's buffer without mercaptoethanol, and were subjected to SDS electrophoresis without boiling, after loading 20 µl of each sample per lane. Samples were run in parallel on 7.5% and 15% SDS-PAGE under non-reducing conditions, after loading 20 µl of each sample per lane. Migration was performed at room temperature using a mini-PROTEAN II system at 25 mA/gel, until the dye front reached the bottom of the gel. The gels were then dried and protein bands revealed using X-OMAT AR. Following autoradiography, the Factor VIII bands of apparent molecular weight 200 and 300 kDa that are consistently hydrolysed by anti-Factor VIII IgG, were scanned so as to allow for the calculation of the rate of hydrolysis of labelled Factor VIII.

Example IV: Fast protein liquid chromatography gel filtration

15

A hundred µl aliquot of anti-Factor VIII IgG of patient Wal (740 µg) treated with 8M urea was subjected to gel filtration on a superose-12 column equilibrated with PBS-0.01% azide at a flow rate of 0.2 ml/min. Five hundred µl fractions were collected and assayed for the presence of IgG by sandwich ELISA and for Factor VIII proteolytic activity after ten-fold dilution. The proteins in fraction 25 were radiolabelled with ¹²⁵I and subjected to SDS-PAGE under non-reducing conditions in parallel with normal polyclonal human IgG. The gel was stained with Coomassie Blue, and also autoradiographed; both images were then overlaid. A major peak was isolated in fraction 25 that corresponded to IgG as indicated by ELISA and SDS-PAGE of the radiolabelled content of the fraction. The hydrolysing activity co-eluted with the IgG fraction and that the activity was not detected in fractions in which IgG was not present (*e.g.*, fraction 35).

30

Example V: Analysis of NH₂-terminal sequences

- Unlabelled human recombinant Factor VIII sucrose formulation (rDNA-BHK) (300 µg, octocog alfa, Bayer Corporation, Berkeley, CA) was treated with the anti-Factor VIII IgG of patient Wal (74 µg) in 1500 µl of 50 mM tris-HCl pH 7.7, 100 mM glycine, 0.025% tween-20 and 0.02% NaN₃ for 24 hours at 38°C. The resultant Factor VIII fragments were run on a 10% SDS-PAGE at 50 mA under non-reducing conditions and transferred for 2 hours at 100 mA on a Hybond-P PVDF membrane (Amersham, Little Chalfont, England) in 10 mM CAPS, 10% ethanol at pH 11.0. After staining with coomassie blue, visible bands were cut and subjected to N-terminal sequencing, using an automatic protein microsequencer Prosize 492 cLC (PE-Applied Biosystems, Foster City, CA). The amount of protein sequenced ranged from 0.5 to 2 pmoles, depending on the fragment.
- The major scissile bonds were as follows: Arg³⁷² - Ser³⁷³ (R³⁷² - S³⁷³), located between the A1 and A2 domains of Factor VIII ; Tyr¹⁶⁸⁰ - Asp¹⁶⁸¹ (Y¹⁶⁸⁰ - D¹⁶⁸¹), located in the N-terminus of the A3 domain ; and Glu¹⁷⁹⁴ - Asp¹⁷⁹⁵ (E¹⁷⁹⁴ - D¹⁷⁹⁵) located within the A3 domain. Multiple site cleavage of Factor VIII by anti-Factor VIII antibodies might originate from individual antibodies with polyspecific catalytic activities or polyclonal populations of antibodies, each exhibiting a unique cleavage site specificity.

Amino acid sequence	Cleavage site
Ser Val Ala Lys Lys His Pro (SVAKKHP)	Arg ³⁷² - Ser ³⁷³ (R ³⁷² - S ³⁷³)
Asp Gln Arg Gln Gly Ala Glu (DQRQGAE)	Glu ¹⁷⁹⁴ - Asp ¹⁷⁹⁵ (E ¹⁷⁹⁴ - D ¹⁷⁹⁵)
Asp Glu Asp Glu Asn Gln Sr (DEDENQS)	Tyr ¹⁶⁸⁰ - Asp ¹⁶⁸¹ (Y ¹⁶⁸⁰ - D ¹⁶⁸¹)

Example VI: Inhibition studies were performed using Pefabloc®, a generic inhibitor of serine proteases

5 Hydrolysis of [¹²⁵I]-Factor VIII by affinity-purified anti-Factor VIII IgG antibodies of haemophilia A patients with inhibitor in the presence of Pefabloc®. [¹²⁵I]-Factor VIII (150 ng) was incubated alone, with 50 µg/ml of immunopurified anti-Factor VIII IgG of patient Wal or in the presence of both
10 anti-Factor VIII IgG and 4 mM of the serine protease inhibitor Pefabloc® (Boehringer) for 5 h at 38°C. Factor VIII was then analysed by 7.5% SDS-PAGE under non-reducing conditions. Following autoradiography, the Factor VIII bands of apparent molecular weight 200 and 300 kDa that are consistently hydrolysed by anti-FVIII IgG, were scanned so as to allow for the calculation of the % of hydrolysis of labelled Factor VIII.

15

The proteolysis of radiolabelled Factor VIII by the anti-Factor VIII allo-antibodies of patient Wal was inhibited to about 62% when the antibodies and Factor VIII were co-incubated in the presence of Pefabloc®, indicating the
20 potency of some serine protease inhibitor to neutralise the catalytic activity of some catalytic antibodies.



CLAIMS

1. A method of determining the presence of anti-Factor VIII allo-antibodies capable of degrading Factor VIII in a mammal, characterised in
5 that it comprises :

- i) isolating the plasma from a sample of blood taken from said mammal,
- 10 ii) isolating anti-Factor VIII allo-antibodies from said plasma ;
- iii) placing said anti-Factor VIII allo-antibodies in contact with Factor VIII for a period of time sufficient to permit any degradation of said Factor VIII by said anti-Factor VIII allo-antibodies ; and
15 iv) determining, after said period of time, whether said Factor VIII has effectively been degraded by said anti-Factor VIII allo-antibodies.

2. The method according to claim 1, characterised in that in step
20 ii), said anti-Factor VIII allo-antibodies are isolated from said plasma by combining them with said Factor VIII, said Factor VIII being preferably coupled to a matrix.

3. The method according to claim 1 or 2, characterised in that in
25 step ii), said anti-Factor VIII allo-antibodies are isolated by affinity chromatography.

4. The method according to claim 3, characterised in that step ii),
said affinity chromatography comprises the use of Factor VIII covalently
30 coupled to a Sepharose matrix, preferably activated with cyanogen bromide.

5. The method according to any one of claims 1 to 4, characterised in that in step iii), said Factor VIII is labelled with a labelling agent, preferably a radio-labelling agent, such as ^{125}I in particular.

5 6. The method according to any one of claims 1 to 5, characterised in that in step iii), said Factor VIII is placed in contact with the anti-Factor VIII allo-antibodies for a period of time of between about 0.5 and about 30 hours, preferably about 10 hours, at a temperature of about 15 to about 40°C, preferably 38°C.

10

7. The method according to any one of claims 1 to 6, characterised in that step iv) is carried out by a determination comprising a separation technique, such as gel electrophoresis, such as SDS PAGE in particular, or gel filtration, such as fast protein liquid chromatography gel filtration in particular, and a visualisation technique, such as autoradiography in particular.

15

8. The method according to any one of claims 1 to 7, characterised in that it further comprises :

20

v) characterising the site(s) in said Factor VIII molecule cleaved by said anti-Factor VIII allo-antibodies.

9. The method according to claim 8, characterised in that said characterisation is carried out by placing said Factor VIII in contact with said anti-Factor VIII allo-antibodies capable of degrading Factor VIII, separating and then sequencing the fragments of Factor VIII resulting therefrom.

25

10. The method according to claim 8 or 9, characterised in that said separation is carried out using a technique such as gel electrophoresis, such as SDS PAGE in particular.

30

11. The method according to any one of claims 8 to 10,
characterised in that said sequencing is carried out using a technique such as
N-terminal sequencing, such as by using an automatic protein microsequencer
5 in particular.

12. The method according to any one of claims 8 to 11,
characterised in that said sequencing locates scissile bonds : Arg³⁷²-Ser³⁷³,
located between the A1 and A2 domains, Tyr¹⁶⁸⁰-Asp¹⁶⁸¹ , located on the N-
10 terminus of the A3 domain, and Glu¹⁷⁹⁴-Asp¹⁷⁹⁵ located within the A3 domain
of the Factor VIII molecule.

13. An amino acid sequence :

15 Ser Val Ala Lys Lys His Pro .

14. An amino acid sequence :

20 Asp Glu Asp Glu Asn Gln Ser .

15. An amino acid sequence :

25 Asp Gln Arg Gln Gly Ala Glu .

16. A peptide or non-peptide analogue of an amino acid sequence of
any one of claims 13 to 15, characterised in that it is capable of inhibiting any
site in the Factor VIII molecule which is susceptible to being lysed by an anti-
Factor VIII allo-antibody.

17. An anti-Factor VIII allo-antibody-catalysed Factor VIII
30 degradation inhibitor.

18. The inhibitor according to claim 17, characterised in that it comprises a protease inhibitor, such as 4-(2-aminoethyl)benzenesulphonyl fluoride hydrochloride in particular.

5

19. The inhibitor according to claim 17 or 18, characterised in that said inhibitor inhibits cleavage of the scissile bonds : Arg³⁷²-Ser³⁷³, located between the A1 domains, Tyr¹⁶⁸⁰-Asp¹⁶⁸¹, located on the N-terminus of the A3 domain, and Glu¹⁷⁹⁴-Asp¹⁷⁹⁵ located within the A3 domain of the Factor VIII molecule.

10

20. The inhibitor according to any one of claims 17 to 19, characterised in that it comprises a peptide or non-peptide analogue of the amino acid sequence :

15

Ser Val Ala Lys Lys His Pro .

21. The inhibitor according to any one of claims 17 to 19, characterised in that it comprises a peptide or non-peptide analogue of the amino acid sequence :

20

Asp Glu Asp Glu Asn Gln Ser .

22. The inhibitor according to any one of claims 17 to 19, characterised in that it comprises a peptide or non-peptide analogue of the amino acid sequence :

25

Asp Gln Arg Gln Gly Ala Glu .

30

23. A pharmaceutical composition characterised in that it comprises a pharmaceutically effective amount of an anti-Factor VIII allo-antibody

capable of degrading Factor VIII, notably as obtainable from the method of any one of claims 1 to 6.

24. Use of anti-Factor VIII allo-antibodies capable of degrading
5 Factor VIII for the preparation of a pharmaceutical composition for the treatment of a mammal suffering from a pathology resulting from abnormal level of Factor VIII in the blood thereof.

25. Use according to claim 24, characterised in that said pathology
10 results from the presence of an excess of Factor VIII in the blood thereof.

26. A pharmaceutical composition characterised in that it comprises
a pharmaceutically effective amount of a Factor VIII degradation inhibitor
according to any one of claims 17 to 22.

15

27. Use of a Factor VIII degradation inhibitor for the preparation of
a pharmaceutical composition, in particular for the treatment of a mammal
suffering from a pathology resulting from the sub-physiological level of Factor
VIII in the blood thereof.

20



ABSTRACT

The present invention relates to a method of determining the presence of catalytic anti-Factor VIII allo-antibodies capable of degrading Factor VIII in a mammal, and of characterising the cleavage sites in said Factor VIII molecule by said catalytic anti-Factor VIII allo-antibodies. It also relates to an anti-Factor VIII allo-antibody-catalysed Factor VIII degradation inhibitor ; and to a pharmaceutical composition comprising said catalytic anti-Factor VIII allo-antibodies which are capable of degrading Factor VIII and which originate from said method of determination ; and further to a pharmaceutical composition comprising said anti-Factor VIII allo-antibody-catalysed Factor VIII degradation inhibitor. Finally, the present invention relates to the application in therapeutics of said anti-Factor VIII allo-antibody-catalysed Factor VIII degradation inhibitor, of a pharmaceutical composition comprising said catalytic anti-Factor VIII allo-antibodies which are capable of degrading Factor VIII and which originate from said method of determination, and of a pharmaceutical composition comprising said anti-Factor VIII allo-antibody-catalysed Factor VIII degradation inhibitor.



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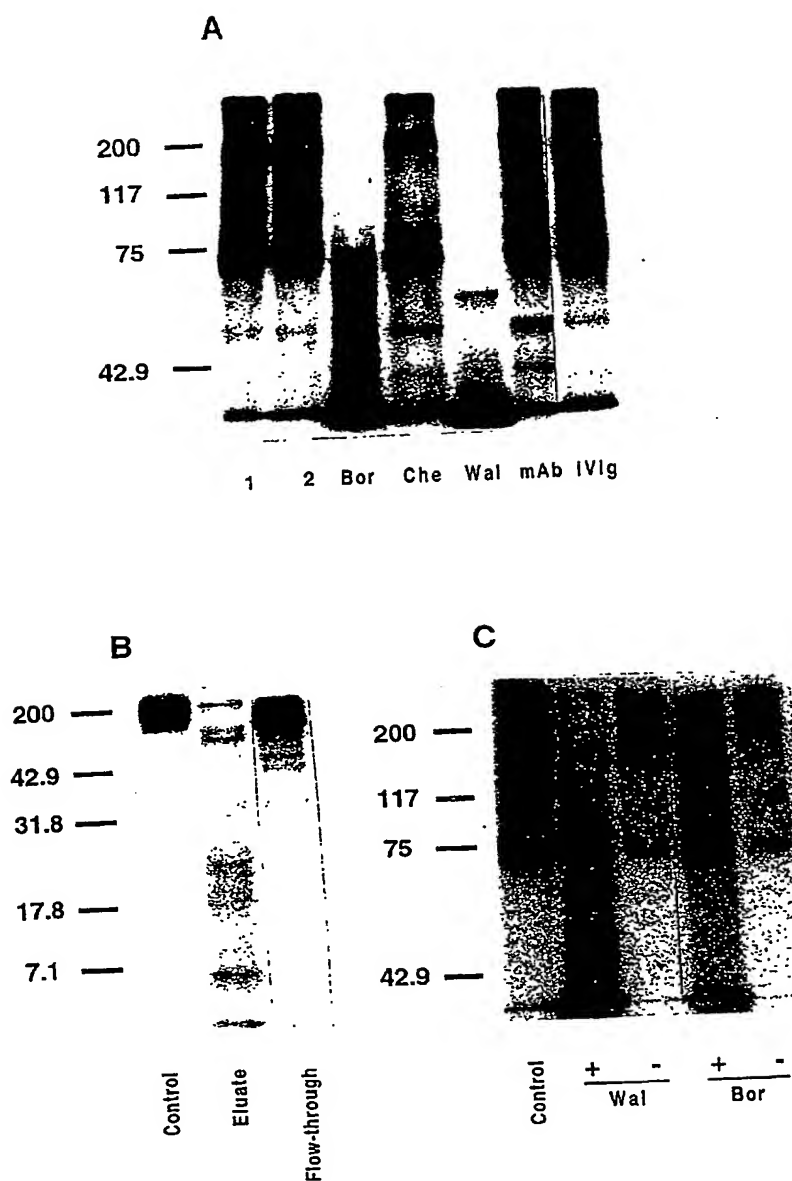


FIG.1

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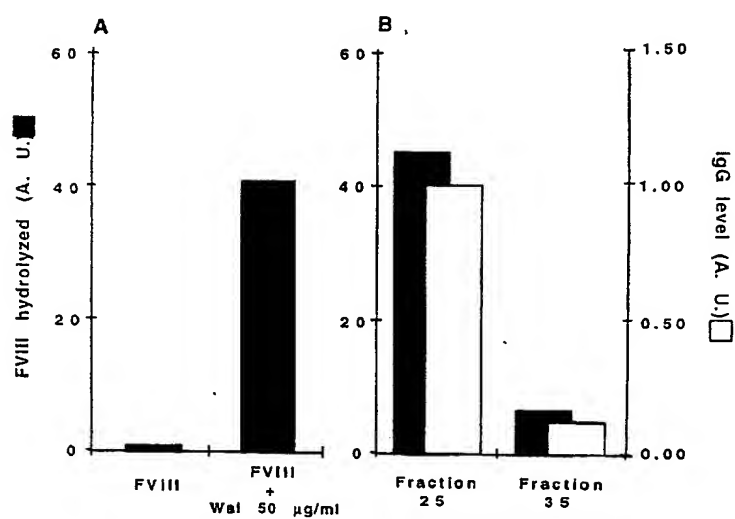


FIG. 2

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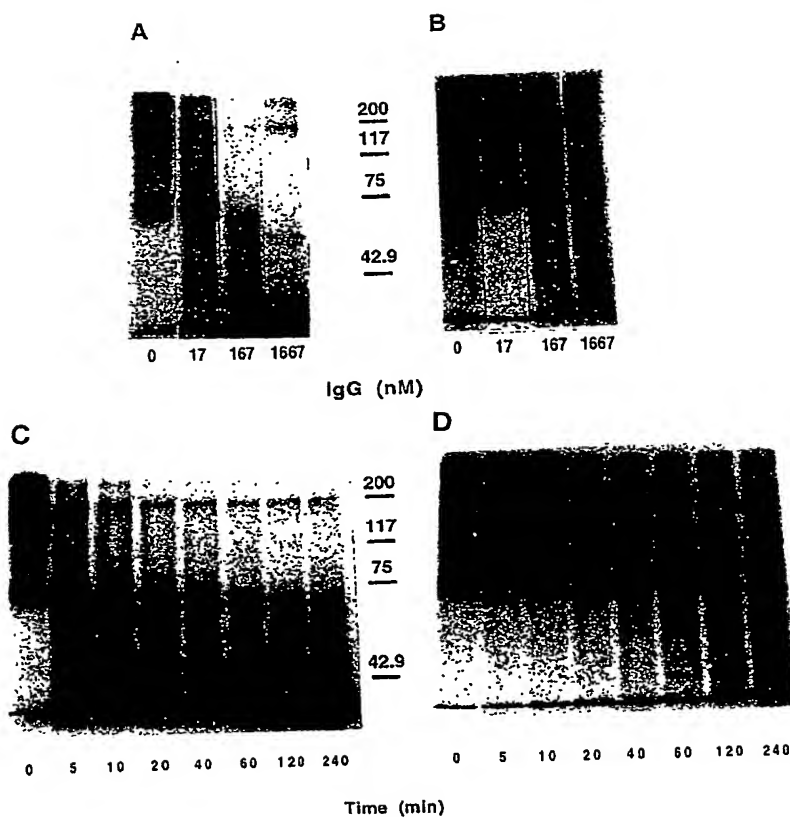
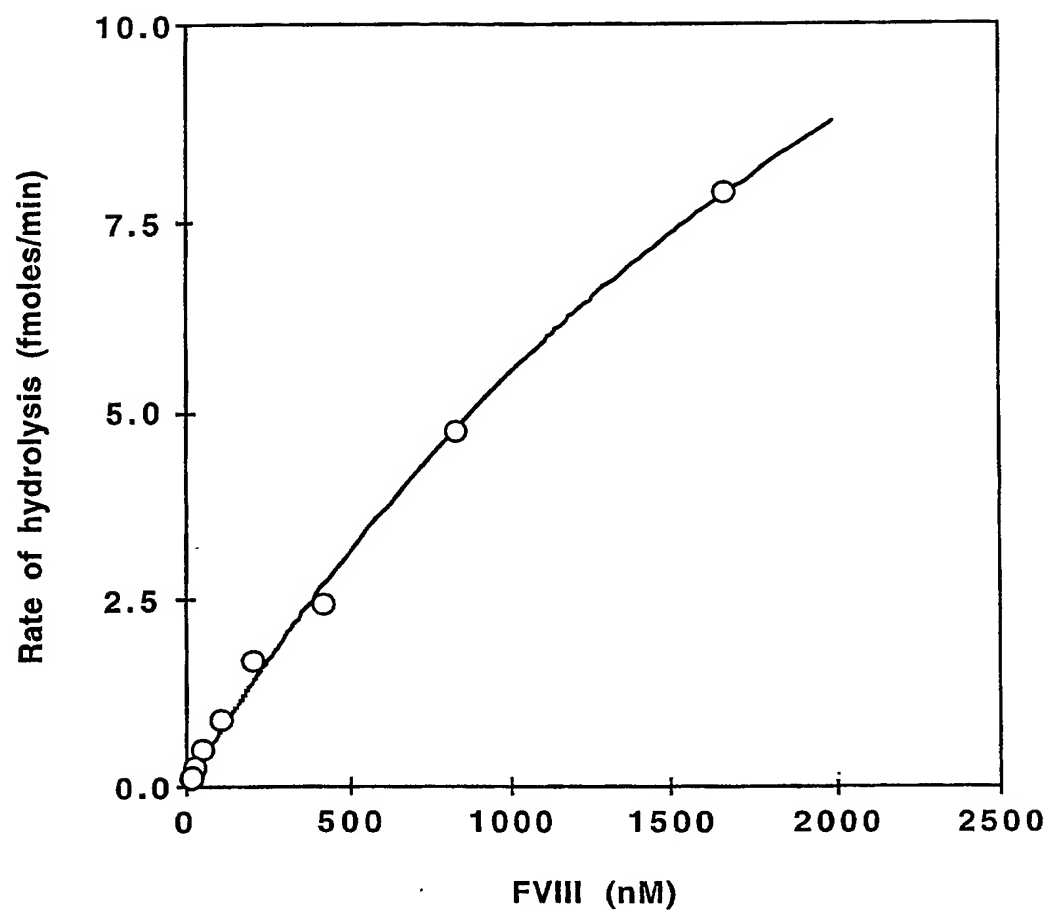


FIG. 3

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K_m (μM)	9.46 ± 5.62
V_{Max} (fmoles/min)	85.1 ± 60.1
Catalytic constant (min^{-1})	0.026 ± 0.018
Catalytic efficiency ($M \cdot \text{min}^{-1}$)	2553 ± 533

FIG. 4

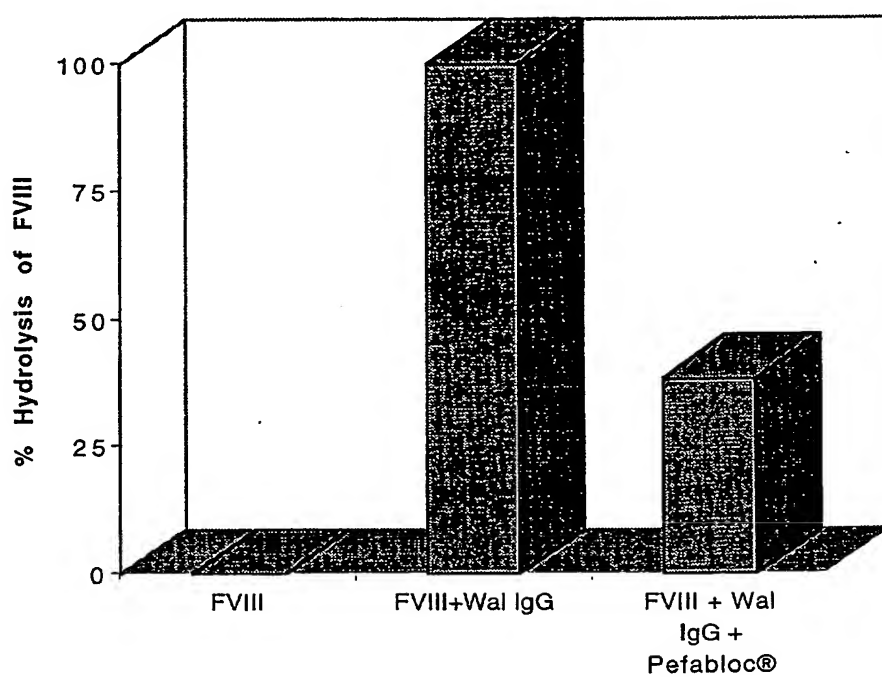


FIG. 5

